Changes in the Cell Wall of the Pear During Ripening

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The cell wall is commonly regarded as an inert background to the metabolism of the plant, although it has been known from the time of Schulze (1892) that cell-wall polysaccharides are used as reserve food materials by many germinating seeds. The ripening of stored fruit is another instance of rapid physiological change in an isolated, non-assimilating tissue, and Barnell (1943) has found a rapid depletion of the hemicelluloses of the ripening banana. Similar but less drastic changes have been reported for the pomaceous fruits by various authors (Smith, 1935; Kidd & West, 1939; Kidd, West, Griffiths & Potter, 1940; Echevin, 1941).

Studies on changes in the cell wall are often difficult to interpret because of the uncertain nature of the fractions which are extracted from it by conventional procedures. 'Pectin', 'hemicellulose' and 'α-cellulose' are the resultants of certain sets of operations rather than chemical individuals. Each of these fractions is normally a mixture of polysaccharides that it is difficult to fractionate further, and the same polysaccharide may be present in more than one fraction.

Hydrolysis of any of these fractions may give a mixture containing as many as seven sugars, and although it is not possible to separate quantitatively the original mixture of polysaccharides, the mixture of sugars can be separated on a paper chromatogram (Jermyn & Isherwood, 1949; Isherwood & Jermyn, 1951) and each estimated quantitatively. If one assumes that each sugar is produced by the hydrolysis of a single polysaccharide, e.g. that all the xylose is obtained originally from xylan no matter which fraction is hydrolysed, then the total amount of any one polysaccharide can be calculated from the amount of the corresponding sugar in the hydrolysis products of the various fractions. This method is open to the criticism that the polysaccharides may contain more than one sugar, but in the pear it has been definitely established that the bulk of the glucose can be accounted for as cellulose, and of the xylose as xylan. These two polysaccharides were isolated in good yield (calculated in terms of the corresponding sugars produced by hydrolysis) from pear cell wall. Pectic acid is known to consist, mainly, if not entirely, of galacturonic acid residues, and araban and galactan of arabinose and galactose residues, respectively (Hirst, 1942; Hirst & Jones, 1938, 1939; Beavan, Hirst & Jones, 1939). The only other sugar present in any amount, mannose, represented only a small proportion of the whole. With the pear cell wall the assumption seems justified that changes in the polysaccharide pattern of the cell wall can be followed by analysing the mixture of sugars produced on hydrolysis. In the present study these methods are applied to give complete analyses of the cell wall of the ripening pear in terms of the polysaccharides corresponding to individual sugar residues.

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MATERIALS

Conference pears grown at East Malling Research Station, Kent, and received during September 1948, were mixed after any damaged or defective fruit had been rejected.

In the main experiment, twelve lots of 50 pears of about the same weight were chosen at random. Each lot was weighed and then the individual pears were placed out of contact with each other on trays lined with waxed Viscose film. The trays were placed on the floor of a light-proof room at +15° and the samples ripened under as nearly identical conditions as possible. The pears were examined at six successive stages, two lots of 50 pears being separately analysed at each stage and the results treated as duplicates. The progress of ripening was assessed by the change in colour of the skin (green to yellow) and by the firmness of the flesh. The latter was measured by the use of an instrument (Penetrometer) which recorded the force (kg.) required to drive a plunger (circular cross section, diameter 1 cm.) into the intact pear.

In subsidiary experiments (cf. Tables 5, 6 and 8) the experimental conditions were varied somewhat from those described above though the general conditions were similar. Any slight variations are mentioned appropriately in the text.

A description of the pears taken at each stage in the main experiment is given in Table 1.

METHODS

Preparation of cell-wall material

Duplicate batches of pears at the appropriate stage in the maturation of the fruit were removed from storage, weighed, and any rotten fruit removed. A correction was applied for

Table 1. *Properties of pears stored at +15° in the dark*

Each lot contained 50 pears, two lots being treated as duplicates. The 'Penetrometer' reading (see text) measured the firmness of the flesh.

Mean wt. per pear in each lot (g.)	Days at +15°	Condition	'Penetrometer' reading (kg.)	Loss of wt. during storage, calc. on original wt. of fruit (%)
161·5	Initial	Green	8·6	—
159·2	—	—	8·7	—
145·1	7	Short of ripe	3·1	4·4
170·5	—	—	3·8	4·5
151·7	11	Yellow, just ripe	2·9	2·6
168·8	—	—	2·8	4·4
165·9	16	Overripe	1·9	4·2
152·0	—	—	2·1	6·1
163·8	23	Sleepy	—	6·3
153·4	—	—	—	10·0
159·1	31	Slushy	—	10·0
158·5	—	—	—	11·2

the loss of material due to the removal of these fruit. The remainder of the fruit in each batch were peeled, cored, frozen overnight at −20° and finally ground to a flour in a rotary rasp, the temperature never rising above about −5°. The weight of dressed material varied between 65 and 75% of the initial weight of the pears, the lower figures being obtained with overripe pears which were difficult to peel satisfactorily. These were peeled over a large vessel so that the expressed juice could be added to the main bulk of the material. The frozen flour was stored in sealed containers at −25°, and before analysis was thoroughly mixed. The frozen flour (500 g.) was added to 2 l. of boiling ethanol and the whole brought to the boil as rapidly as possible and maintained at the boiling point for 30 min. The mixture was filtered through a sintered-glass funnel, and the residue extracted with several changes of boiling 95% (v/v) ethanol until the filtrate gave a negative anthrone test for carbohydrates (Dreywood, 1946). The final product was dried in a current of air. Yield, 15–18 g. from each batch.

The cell-wall material prepared in this way contained all the polysaccharides originally present in the cell sap. These were mostly members of the pectic group of substances.

Fractionation of cell-wall material

To provide an overall picture of the analysis to which each sample of cell wall was subjected, a typical example is given in Table 2.

The ethanol-insoluble polysaccharides were first fractionated by extracting all the material soluble in hot water (this can be regarded as roughly equivalent to the 'pectin' of earlier workers). The insoluble residue was delignified by treatment with sodium chlorite, and the polysaccharides remaining ('holocellulose') were fractionated by extracting with N- and 4N-KOH.

This left a final residue of polysaccharides which were not fractionated further ('α-cellulose'). At all stages, the various products were analysed for ash, nitrogen, methoxyl and lignin, and, after hydrolysis, for their constituent sugars. The results were summed so as to obtain a comprehensive picture of the composition of the cell wall and to detect the presence of unknown compounds.

The details of the various extraction and hydrolysis procedures are as follows.

Material soluble in hot water. The cell-wall preparation (10 g.) was extracted with 750 ml. of water at 98–100° for 12 hr. in a boiling-water bath. The mixture was filtered through a sintered-glass funnel and the residue washed with 6 × 200 ml. of boiling water. The residue was thoroughly mixed with acetone and washed on the filter with more acetone. It was then dried in a current of air. Yield of insoluble material, 6·5–7·5 g. Addition of such salts as ammonium oxalate to the extracting liquor did not lead to any significant increase in the amount of material removed.

The filtrate and washings from the extraction described above were concentrated under reduced pressure, at a maximum bath temperature of 40°, to a final volume of 75 ml. The polysaccharides were not precipitated from this concentrate by the addition of ethanol because earlier experiments had shown that the araban component is extensively hydrolysed by the extraction procedure and would not be precipitated. The whole concentrate was therefore used for the subsequent analysis.

Holocellulose. The insoluble residue remaining after the water-soluble material had been extracted was delignified by the method described by Wise, Murphy & D'Addieco (1946) for wood.

The material (5 g.) was suspended in 160 ml. of water in a 250 ml. Erlenmeyer flask, 10 drops of acetic acid and 1·5 g. of sodium chlorite were added and the flask was immersed to a point above the level of the liquid inside in a water bath at 75°. The flask was closed by a loose-fitting glass bubble. At hourly intervals 10 drops of acetic acid and 1·5 g. of sodium chlorite were added. After treatment for 4 hr. (the lignin content had fallen from 16% to 2–3%) the contents of the flask were filtered through a small sintered-glass funnel. The flask was washed with several portions of ice water to remove adhering matter, and the contents of the funnel were washed repeatedly (about 10 times) with ice water, and then with acetone. The powder was dried in a current of air. The yield of holocellulose appeared to increase as delignification proceeded. A typical experiment is described in Table 3. The apparent rise of about 5% in the amount of the holo-

Table 2. *Fractionation of cell-wall material from Conference pears ripened for* 16 *days at* +15°

Figures are calculated as percentage of initial wt. of alcohol-insoluble material. Sugars are expressed in the anhydro form.

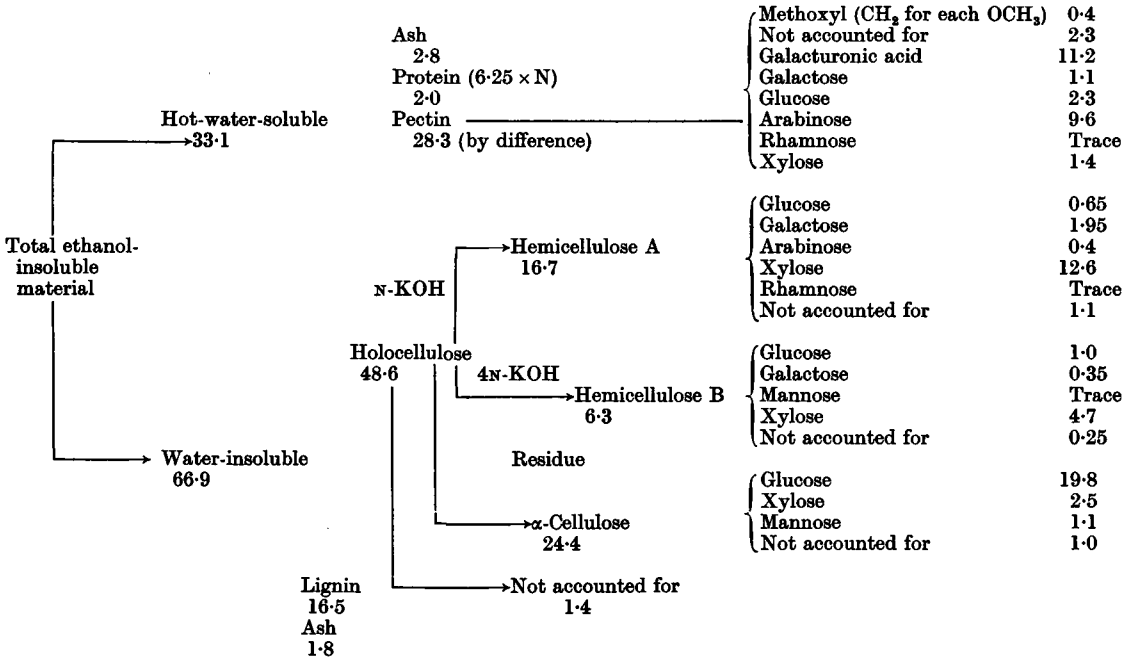

Table 3. *Yield of holocellulose from pear cell wall*

Alcohol-insoluble material treated with sodium chlorite (Wise *et al.* 1946) to remove lignin (see text).

Time of extraction (hr.)	Yield of delignified residue (%)	Lignin remaining in residue (%)	Apparent holocellulose (cell wall less lignin) (%)
0	100	24·5	75·5
1	95·0	16·4	78·6
2	90·4	10·7	79·7
4	82·1	2·1	80·0

cellulose occurred in all the samples examined. It seems to be due to the inherent defects of hydrolytic methods of estimating lignin. These lead to condensations between lignin and various substances in the cell wall (especially protein) and result in too high a figure for lignin.

The extraction liquors from the procedure described above gave only very faint tests for carbohydrate by the sensitive anthrone method (Dreywood, 1946) so that the loss of polysaccharide material was negligible.

Hemicellulose A. The holocellulose was fractionated according to the method of Wise *et al.* (1946) with the modification that the alkali extractions were not carried out under a stream of N_2. It was found that if the extractions were made in small Erlenmeyer flasks filled very nearly to the top with the extracting solution, the modification did not affect the results and the procedure was more convenient when a large number of analyses had to be made.

The holocellulose (2·5 g.) was placed in a 125 ml. Erlenmeyer flask and 100 ml. of N-KOH added. The flask was closed with a rubber bung and placed at a constant temperature of 20°. The contents were swirled gently at 10 min. intervals for 2 hr. The mixture was filtered through a sintered-glass funnel, washed with 50 ml. of N-KOH and then 150 ml. of water. The filtrate and washings were run into a suction flask, containing 10 ml. of acetic acid. This solution was transferred to a 2 l. flask and the suction flask rinsed twice with 25 ml. of water. The combined filtrates and washings were then diluted to 2 l. with ethanol and allowed to stand overnight.

The white flocculent precipitate was centrifuged, and then washed on the centrifuge successively with ethanol, acetone and ether. If the dehydration had been carried out properly the product was a light fluffy powder. This contained about 80% of the lignin that was originally present in the holocellulose.

Hemicellulose B. The residue after the extraction of the hemicellulose A was returned to the 125 ml. Erlenmeyer flask and 100 ml. of 4N-KOH added. The flask was placed at a constant temperature of 20° and swirled gently at 10 min. intervals for 2 hr. After the treatment, the liquid was filtered through a sintered-glass funnel and the residue washed with 25 ml. of 4N-KOH, 250 ml. of water and 25 ml. of 2N acetic acid. The filtrate and washings were run into a suction flask containing 45 ml. of acetic acid. The hemicellulose B was isolated from this solution in the same manner as for hemicellulose A described above.

α-Cellulose. This was the residue after the extraction of the hemicellulose B.

Hydrolysis of cell-wall fractions

Material soluble in hot water. The concentrated extract (75 ml.), to which had been added about 0·05 g. of urea nitrate, was heated in a boiling-water bath under reflux. When the temperature of the solution reached that of the bath, 3 ml. of colourless HNO_3 (sp.gr. 1·57) was added slowly with shaking. It was important not to add the acid till the contents of the flask were hot, as otherwise a considerable amount of the polysaccharide material precipitated and only redissolved after protracted heating. The acidified extract was heated for 12 hr. in the boiling-water bath, and then cooled. Tests showed that the last trace of digalacturonic acid disappeared after hydrolysis for 12 hr., though the maximum yield of galacturonic acid was produced after 10 hr. and was about 2% higher than the value at 12 hr. The recoveries of various sugars treated in the same way were arabinose 92%, galactose 97%, and glucose 96%. The recovery of galacturonic acid by this method, compared with that produced by total hydrolysis with a polygalacturonidase from the mould *Botrytis*, showed that 91 and 89% were produced at 10 and 12 hr. respectively (Jermyn & Tomkins, 1950).

The recoveries of the aldose sugars in the presence of galacturonic acid were rather less than the figures given above, but not by any definite amount. It was found in practice that the sugars recovered from the hydrolysis of the water-soluble fraction averaged about 90% of the amount expected, but because the recoveries of the various sugars were not sensibly constant no attempt was made to apply correction factors. The results for this fraction will therefore be low by about 10%.

Hemicelluloses. The hemicellulose fraction (400 mg.) was mixed with 10 ml. of 0·5 N-HNO_3 containing a few crystals of urea nitrate in a test tube closed with a glass bubble and the whole heated in a boiling-water bath for 4 hr. With hemicellulose A a little undissolved material remained, but tests showed that this was not carbohydrate and that it was probably connected with the lignin known to be present in the original material.

α-Cellulose. To 500 mg. of α-cellulose was added 5 ml. of 72% (w/w) H_2SO_4 in 0·5 ml. portions, the mixture being thoroughly stirred after each addition. After standing at room temperature for 2 hr., the α-cellulose dissolved completely. The solution was then poured into 360 ml. of water (including any washings necessary to effect a quantitative transfer of the solution) and the whole boiled under reflux for 12 hr. At the start the liquid was often faintly opalescent, but this soon disappeared, and at the end the amount of material remaining undissolved was less than 5 mg. and consisted almost entirely of ash. A few drops of a 1% solution of methyl red were added and then 15 g. of $BaCO_3$. The mixture was heated in a boiling-water bath, with constant shaking, until it was neutral to methyl red, and then filtered while hot through a sintered-glass funnel. The barium precipitate was washed exhaustively with boiling water (6 × 50 ml.) and the combined filtrate and washings were evaporated *in vacuo* to about 15 ml. A few drops of 0·1 N-H_2SO_4 were added at the start of the distillation and an occasional addition was made subsequently to keep the solution neutral to methyl red. This was found necessary to prevent the isomerization of any aldopentoses into ketoses in the slightly alkaline (pH 8) solution left after neutralization with $BaCO_3$. Xylose and arabinose are particularly susceptible.

Analysis

Sugars in the hydrolysis products. These were separated and estimated by the method described earlier by Jermyn & Isherwood (1949) with a paper chromatogram. An important improvement to this method has recently been described by Wager (1954). The hydrolysate from the α-cellulose was used directly but the nitric acid in the other hydrolysates (hemicellulose and water-soluble material) had to be removed before they could be examined on a paper chromatogram. The general procedure was as follows. The solution of sugars, after hydrolysis, was neutralized with 2 N-KOH and evaporated to dryness *in vacuo*. A mixture of absolute ethanol and benzene was distilled through the residue to remove any traces of water remaining, and the thoroughly dried residue was extracted with absolute ethanol (in which KNO_3 is almost insoluble) until all reducing material had been removed. The alcoholic solution was decolorized with a little charcoal, and evaporated to dryness *in vacuo*. With the hydrolysates from the water-soluble material (pectin) a slight modification was used. The dried residue was extracted with acetic acid instead of ethanol, filtered from suspended KNO_3 and the sugars were precipitated by the addition of anhydrous ether (5 vol.). The syrupy precipitate was redissolved in acetic acid and again precipitated with ether. The residue of sugars obtained by either method was dissolved in an appropriate volume of water to give a 2% solution suitable for chromatography.

Compounds other than polysaccharides in the cell wall. These were determined on each fraction and the figure for the carbohydrate content was corrected appropriately. There is almost certainly some error involved in this procedure. It is known, for example, that the water-insoluble protein present in the cell-wall material is destroyed by chlorite oxidation, and in some cases contributes as much as 10–20% to the apparent lignin figure. A similar state of affairs is probably true of other constituents, so that the corrected figure for the carbohydrates can be regarded only as approximate. The following constituents were determined: lignin (Ritter, Seborg & Mitchell, 1932), methoxyl (Hickinbottom, 1936), nitrogen ((Kjeldahl) Markham, 1942), volatile materials by estimating the loss in weight after drying at 105° for 12 hr., and ash by incineration at red heat.

Typical analysis. The figures obtained for the various sugars produced by the hydrolysis of the cell wall were recalculated to give the amounts of anhydro sugars originally present in the cell wall. The anhydro sugar (hexose or pentose minus one molecule of water) was regarded as being the equivalent of the appropriate polysaccharide. The figures were all based on the original fresh weight of the pears, and the anhydro sugars were summed irrespective of the fraction of the cell wall examined. The only exception was the glucosan removed by hot water; this has been treated as if it were entirely starch, and has been excluded from the cell-wall totals. There is almost certainly some error in this procedure but the results can be compared with those of other workers (Kidd *et al.* 1940) who determined the cell wall of pear as 'alcohol-insoluble material other than starch', and recorded loss of weight after treatment with a crude diastase preparation as a measure of starch content. Two assumptions have been made in calculating the amounts of the various polysaccharides in the original fresh fruit from the amounts of the anhydro sugars in the derived cell wall.

Perhaps the most important is that the flesh and skin removed with the peel and cores was identical with that retained in the dressed fruit. An analysis of a sample of pear peelings compared with the corresponding analysis on the dressed fruit is given in Table 4. The figures show that the composition is very much the same, any observed differences being of less consequence in practice because the peelings form only 15–20% of the total weight of the fruit. The other assumption was that the loss in weight during storage was uniformly distributed throughout the fruit.

RESULTS

Qualitative and quantitative composition of the alcohol-insoluble polysaccharides

The sugars produced on hydrolysis of all the arbitrary fractions—pectin, hemicellulose A and B, and α-cellulose—were glucose, xylose, arabinose, galactose, galacturonic acid and rhamnose (a trace). The rhamnose, for which no analytical figures are given, was never present in any fraction in more than trace amounts, and it formed at the most perhaps 1% of some pectin fractions. A uronic acid could be shown to be present in the hemicellulose fractions but could not positively be identified on the paper chromatograms. Examination of a purified xylan prepared from these fractions (Chanda, Hirst & Percival, 1951) showed that it contained one glucuronic acid terminal unit to a chain of 115 xylose units, and the inference is that the uronic acid present in the hemicellulose fractions was probably glucuronic acid.

A study of the composition of each of the arbitrary fractions throughout the storage of the fruit showed that there were no consistent ratios between the sugar residues present, such as would have been expected if the fractions represented definite components of the cell wall. It appears that each stage of the extraction procedure removes from the cell wall a complex mixture of polysaccharides, the composition of which is governed by the previous history of the sample. An illustration of this is given in Table 5.

Table 4. *Analyses of the ethanol-insoluble polysaccharides from the combined peelings of 200 pears*

The pears (mean wt. 160 g.) had been stored for 11–23 days at +15° and were just ripe to overripe in condition. The figures are compared with those for dressed pears (mean wt. 165 g.) which had been stored for 11 days.

Constituent	Composition of ethanol-insoluble material: From dressed pears (%)	From peelings (%)
Glucosan (not starch)	21·4	17·6
Starch	2·5	0·2
Galactan	3·5	1·6
Mannan	1·1	3·7
Xylan	21·0	31·0
Araban	10·0	8·4
Polygalacturonic acid	11·5	6·4
Lignin	16·1	23·5
Ash	4·6	1·4
Not accounted for	8·3	6·2

Table 5. *Relative proportions of different polysaccharides in the arbitrary fractions (pectin, hemicelluloses and α-cellulose) extracted from the ethanol-insoluble material of unripe and ripe Conference pears*

Unripe pears were analysed as received from orchard, and ripe pears after storage at +15° for 14 days. Each sample contained 70 pears, mean wt. of each pear 166 g. Six samples were analysed in each group. Range of values given in parentheses.

Arbitrary fraction	Polysaccharides present	Condition of fruit: Unripe	Ripe
Pectin	Glucosan*	0·24 (0·12–0·82)	0·35 (0·13–1·3)
	Galactan	0·29 (0·20–0·46)	0·33 (0·26–0·40)
	Xylan	0·08 (0·055–0·12)	0·02 (0·00–0·06)
	Araban	0·78 (0·62–1·05)	1·40 (1·22–1·51)
	Polygalacturonic acid	1·00	1·00
Hemicellulose A	Glucosan	0·20 (0·15–0·24)	0·07 (0·05–0·08)
	Galactan	0·085 (0·05–0·12)	0·04 (0·02–0·05)
	Xylan	1·00	—
	Araban	0·12 (0·08–0·15)	0·02 (0·015–0·03)
	Uronic acid	0·006 (0·004–0·008)	0·001
Hemicellulose B	Glucosan	0·47 (0·30–0·60)	0·21 (0·18–0·26)
	Galactan	0·20 (0·14–0·27)	0·12 (0·10–0·13)
	Mannan	0·07 (0·05–0·10)	0·045 (0·025–0·06)
	Xylan	1·00	1·00
α-Cellulose	Glucosan	1·00	1·00
	Galactan	Trace	Trace
	Mannan	0·14 (0·12–0·15)	0·02 (0·0–0·04)
	Xylan	0·02 (0·0–0·05)	0·12 (0·08–0·14)

* This is soluble glucosan, possibly starch or starch-degradation products. The amount is very variable.

The various fractions (pectin, hemicelluloses and α-cellulose) from six samples (each contained 70 pears, mean weight 166 g.) of ripe and unripe pears were analysed and the results expressed in the form of ratios, the polysaccharide present in largest amount being equated to unity. This method of presentation was used so as to bring out more clearly the variation in the composition of the arbitrary fractions. The six samples of unripe pears were selected from the cases of pears immediately they arrived at the laboratory from the orchard and the six samples of ripe pears after the fruit had been matured for 14 days at +15° (their condition was roughly equivalent to that of the fruit in the main experiment after 11 days, cf. Table 1).

The figures show that the composition of each fraction is very variable, even when samples are at the same stage of maturation, and that the composition of the corresponding fraction changes markedly when unripe pears are compared with ripe. Other experiments have shown that the same is true when pears are compared that have been picked at the same stage of growth and from the same trees in two different seasons (1947 and 1948). Since the composition of the various fractions is not even approximately constant, they cannot be considered as fundamental components of the cell wall.

A much more regular and consistent picture is obtained if the composition of the cell wall is considered in terms of the constituent polysaccharides. The assumption is made that each polysaccharide is built up from only one sugar and that the polysaccharides may appear in a number of fractions. Some results on unripe and ripe pears which have already been partly described in Table 5 are given in terms of the constituent polysaccharides in Table 6.

The glucose produced by the hydrolysis of the water-soluble fraction is assumed to be derived from a different polysaccharide from the glucosan in the holocellulose. Starch is known to be present in the fruit at certain stages of growth, and the soluble glucosan may well be derived from starch.

In the experiments on the effect of ripening on the composition of the pear fruit which are described below, the analytical figures have been expressed in terms of the derived polysaccharides, as in Table 6, and changes in the amounts and composition of the arbitrary fractions pectin, hemicellulose A and B and α-cellulose have been ignored.

Table 6. *Total amount of each polysaccharide in the pear cell wall (minus the soluble glucosan)*

The mean figure, together with the standard error, has been calculated for each polysaccharide from the facts presented in Table 5 (for details of experimental conditions see Table 5).

Polysaccharide	Amount of each polysaccharide (g./pear) Unripe	Ripe
Holocellulose glucosan (cellulose)	0·98±0·065	0·96±0·00
Galactan	0·266±0·03	0·17±0·025
Mannan	0·126±0·008	0·03±0·02
Xylan	0·745±0·06	0·955±0·11
Araban	0·54±0·041	0·48±0·05
Polygalacturonic acid	0·64±0·08	0·34±0·05
Total	3·6±0·20	3·16±0·24

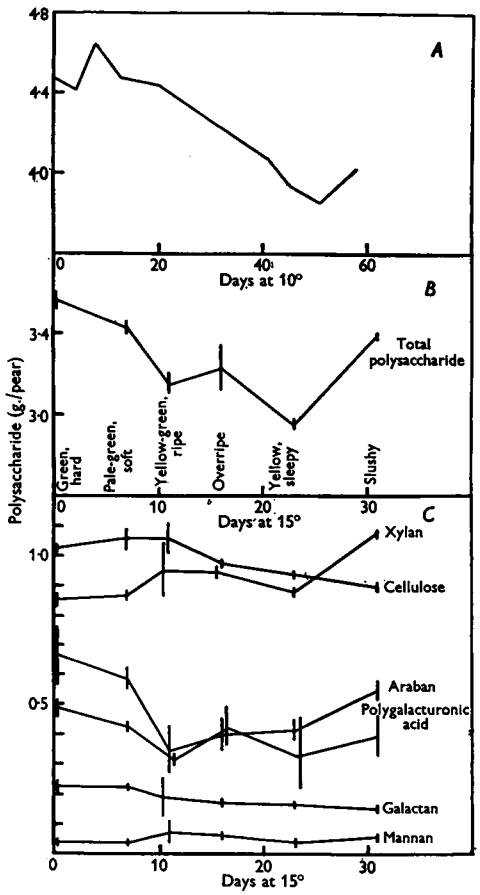

Fig. 1. Changes in the polysaccharide constituents of Conference pears during ripening at +15° and at +10°. The material included polysaccharides which were insoluble in 80% ethanol, such as pectin. *A* represents the changes in the total polysaccharides (including protein, lignin, methoxyl and ash) in pears stored at +10°, and is calculated from the data of Kidd *et al.* (1940). *B* represents the changes in the total polysaccharides in pears stored at +15°, recorded in the present study. *C* represents the changes in the individual polysaccharides corresponding to *B*. *A* has been drawn so as to be roughly comparable with *B* and *C*. The condition of the pears for culinary purposes is indicated by the appropriate description in *B*. The length of the vertical lines indicates the scatter between duplicate estimations.

Table 7. *Changes in the degree of methylation of polygalacturonic acid during storage of pears at +15°*

Figures were calculated from the analyses of the 'pectin' fraction obtained in the main experiment (see text and Tables 1 and 2).

Days of storage	0	7	11	16	23	31
Ester methoxyl (OMe)	2·1	1·5	1·2	0·9	0·5	03·5
(% of cell wall)	1·8	1·9	Nil	0·4	0·5	0·7
Polygalacturonic acid plus OMe as CH_2	11·2	10·2	7·6	11·6	10·3	10·4
(% of cell wall)	10·3	10·6	6·4	8·2	5·4	7·4
Methoxyl content (OMe) of methylated	19·0	15·0	15·7	8·0	4·7	3·4
polygalacturonic acid (%)	17·7	18·3	Nil	4·7	9·6	9·3

Changes in the ethanol-insoluble polysaccharides of pears stored at +15°

The observed changes in the principal polysaccharides present in pear fruit are given in Fig. 1. The range of values between the duplicate estimations are indicated by the extremities of the vertical lines. The value for each point has been corrected for ash, moisture, methoxyl, lignin and nitrogen content in the original fractions and for the loss of sugar during hydrolysis. Most accurately the curves should be described as those for the various anhydro sugars, but it has been assumed, as described previously, that this is equivalent to a first approximation to the corresponding single sugar polysaccharides. In two instances, such polysaccharides, xylan and cellulose, have been isolated in good yield from the appropriate fractions, thus confirming the general correctness of the assumption. The isolation will be described later.

The curves are the mean of two sets of results, which in general follow each other closely. The widest scatter occurs at the stage corresponding to the onset of ripening. This probably means that, at and about the climacteric, such rapid changes are taking place that it is impossible to get samples at exactly equivalent stages of change. There may also be considerable variations in the exact course of the changes through factors which cannot easily be controlled. For example, if an ethylene-producing pear arises at an early stage in the storage of a sample, it will bring on a premature climacteric in adjacent pears and thus anticipate the changes which occur at a later stage in the other samples.

The methoxyl content of the polysaccharides falls during the storage of the pear, and this fall can be accounted for by a fall in the methoxyl content of the pectin fraction. The actual degree of esterification of the polygalacturonic acid in the pectin fraction falls during storage of the fruit. In Table 7 the estimated methoxyl and polygalacturonic acid contents of the pectin fractions are combined to give the methoxyl content of the methylated polygalacturonic acid. Practically all the polygalacturonic acid is extracted in the pectin fraction.

The figures are very variable but indicate that during storage methyl groups are lost from the polygalacturonic acid fraction. Comparison with a fully esterified polygalacturonic acid (methoxyl 16·3 %) indicates that at the start the polygalacturonic acid was probably fully esterified.

Changes in the ethanol-insoluble polysaccharides of pears stored at +5°

Pears stored at lower temperatures ripen more slowly and in some cases develop abnormally. It was of interest, therefore, to examine the changes in the polysaccharide constituents as compared with those in pears ripened at +15°, when both were in approximately the same physical state as judged by colour, firmness and eating quality. The results of an experiment, in which pears stored at +15° for 23 days (sleepy stage of overripeness) were compared with the original unripe pears and with pears stored at +5° for 84 days, are given in Table 8.

The pears at +5° ripened normally and it is noteworthy that the changes in the polysaccharide constituents closely paralleled those at the higher temperature.

Changes in the minor constituents of the cell wall

There are indications of changes in the lignin content during the ripening process which seem to parallel those in the cellulose but, since the estimation of lignin is unreliable and there is considerable variation between duplicates, it is uncertain how far these changes are significant. The observed changes may in fact merely reflect changes in the polysaccharide components which contribute to the production of the insoluble lignin by the method used (72 % H_2SO_4). The figures for lignin, as well as those of ash and nitrogen, have been used to calculate the absolute amounts of polysaccharide in the cell wall, and have not been investigated separately.

Isolation of polysaccharides from main fractions of cell wall

The basic assumption in all the analyses described above has been that, to a first approximation, all the polysaccharides in the cell wall are built up from one

Table 8. *Comparative analysis of the polysaccharides of pears stored at +15° and +5° until they were overripe*

Two samples of pears (50 pears in each, mean wt. of pears 141 g.) were stored in closed containers at +5° for 84 days. They were then in the 'sleepy' stage of overripeness. The figures for the unripe and those ripened at +15° for 23 days are taken from the results of the main experiment (see Tables 1 and 2). Figures are given for each of the duplicates.

	Amount of each polysaccharide (g./pear)					
	Original unripe		After storage at +15° for 23 days		After storage at +5° for 84 days	
Polysaccharide						
Holocellulose glucosan (cellulose)	1·03	1·03	0·92	0·95	0·75	0·75
Galactan	0·24	0·21	0·17	0·16	0·1	0·085
Mannan	0·05	0·03	0·03	0·05	0·03	0·03
Xylan	0·84	0·87	0·87	0·88	0·72	0·75
Araban	0·58	0·76	0·37	0·45	0·25	0·24
Polygalacturonic acid	0·51	0·46	0·45	0·22	0·31	0·37
Soluble glucosan	0·19	0·27	0·13	0·13	0·06	0·04

sugar residue only. Some of the polysaccharides such as polygalacturonic acid, araban and galactan have been isolated from similar fractions of other plant tissues and shown to be of this type, so that it can be assumed with a reasonable degree of certainty that the same is true of the pear. The composition of the hemicellulose and α-cellulose fractions is not so clear, and in the present study these have been examined in detail. From the hemicellulose fractions a pure xylan has been prepared as follows, in good yield calculated on the amount of xylose in the hemicellulose fraction.

Hemicellulose A and B (70 g., extracted from holocellulose with 4N-KOH, as described under Analysis) was dissolved in 4N-KOH and to this solution was added 3·5 l. of a solution (a modified Benedict's reagent) containing 162 g. of potassium citrate, 130 g. of K_2CO_3, and 17·3 g. of $CuSO_4,5H_2O$ per litre. The flask was stoppered, shaken vigorously to coagulate the precipitate and allowed to stand overnight. The deep-blue xylan–copper complex was centrifuged and washed once with 5 l. of N-KOH. The complex was decomposed by stirring into 4 l. of acetic acid. No trace of the blue colour of the complex remained. The mixture was centrifuged and the precipitate of xylan washed on a sintered-glass funnel with 80% (v/v) acetic acid until all the copper had been removed (about 1·5 l. was required). The xylan was washed successively with glacial acetic acid, acetone and ether. Yield, 38 g., 71% of the analytically determined xylan content of the hemicellulose fractions. Hydrolysis with 0·5N-HNO_3 gave xylose 90%, glucose 7%, galactose 3% and a trace of arabinose. To 30 g. of this crude xylan dissolved in 1 l. of N-KOH was added 1 l. of the Benedict's reagent described above, and the mixture was allowed to stand for 2 hr. It was centrifuged, washed on the centrifuge with 2 l. of N-KOH and the precipitate added to 2·5 l. of glacial acetic acid. The precipitated xylan was washed free of copper with 80% acetic acid, then treated with acetone and ether, and air-dried. Yield, 24 g. Hydrolysis showed that not more than 1% of sugars other than xylose were present. A repetition of the purification procedure gave a pure xylan, ash by incineration 0·4%, $[\alpha]_D^{20} - 76°$ in N-NaOH (*c*, 1), Cu 0·018%. Hydrolysis with 0·5N-HNO_3 gave crystalline D-xylose in 91% yield (Heuser & Jayme, 1923), and from the mother liquor was isolated (as crystalline xylose dibenzylidenedimethylacetal) a further 4% (Breddy & Jones, 1945). Total yield, 95%.

The isolation of a degraded cellulose from the α-cellulose obtained from pear cell wall has already been described (Hirst, Isherwood, Jermyn & Jones, 1949). From 30 g. of the α-cellulose, 7·5 g. of the degraded cellulose was obtained. A similar yield of a degraded cellulose was obtained when cotton cellulose was used as starting material, and it appears that the glucose residues in the α-cellulose fraction of pear cell wall are present in the form of the polyglucosan cellulose. The properties of the degraded cellulose from pear cell wall were identical with those of a degraded cellulose obtained in the same way from genuine cotton cellulose.

DISCUSSION

Before the results obtained on pear fruit that have been picked and held in storage are analysed, it is important to know what has happened to the cell wall in the immediate period before the fruit is picked. With the Kieffer pear, Smith (1935) found that the amount of cell-wall material changed very little after a certain point in the natural growth on the tree, and that the increase in size of the fruit was mainly due to water and sugars. In the present study it has been found that the same appears to be true of Conference pears. In Table 9 some figures are given for the cell-wall material of Conference pears picked in different seasons and at different stages of maturity.

The figures indicate that the mean weight of the cell-wall material per pear is much the same whether the fruit are picked early (60 g. in weight) or late (161 g. in weight), and suggest that the cell wall at this time is largely static in nature. This, however, may be a reflexion of a balance between the rate of breakdown of carbon compounds due to respiration and the supply of nutrients from the tree. After picking, the respiration can occur only at the expense of the sources of energy present initially in the fruit, and any balance which may

Table 9. *Absolute weight of cell-wall material per pear at different periods of growth of Conference pears*

The figures are averages from samples of at least 50 pears and include lignin, protein and ash, but not starch.

Source of data	Date of picking	Mean wt. of one pear (g.)	'Alcohol-insoluble content less starch' (%)	Mean wt. of cell-wall material per pear (g.)
Kidd *et al.* (1940)	4. viii. 36	60·2	7·85	4·72
	25. viii. 36	82·6	5·40	4·46
	16. ix. 36	117·5	3·81	4·56
Present study	21. ix. 48	161·5	2·99	4·82
		159·2	2·90	4·62
	20. x. 47	154·0	2·68	4·13

exist in the fruit on the tree is completely changed. If the cell wall is linked with the metabolism of the fruit then this change will affect the composition of the cell wall progressively with time.

Examination of the results shown in Fig. 1 (including those of Kidd *et al.* 1940) indicates that the cell wall is not static but both loses and gains in amount during storage.

The fall in the total polysaccharides is interpreted to mean that at least a part of the carbon compounds locked up in the cell wall became available for conversion into carbon dioxide. The extent to which this occurs depends upon the temperature of storage. Overripeness and tissue breakdown occur more rapidly at higher temperatures and probably set a limit to the orderly breakdown of the cell wall. At + 5° the depletion of the cell wall proceeds further than at + 15°, though at a slower rate. The labile material appears to be mainly araban. The low-temperature storage experiment in particular (Table 8) shows a large loss of this polysaccharide, accompanied by significant losses in galactan and holocellulose glucosan. The decrease in the amounts of these polysaccharides during storage suggests very strongly that the cell wall can act as a reserve substance for the respiration of the cell; the sharp rise at the stage of overripeness indicates that the relationship between the cell wall and the cytoplasm involves synthesis of polysaccharides as well as breakdown. In fact, the cell wall appears to be in dynamic equilibrium with the cytoplasm, and the apparently static nature of the cell wall in the fruit on the tree does not reflect its full relationship with the cytoplasm.

The actual changes that occur in the polysaccharide constituents are calculated in terms of the whole pear, though they are probably mostly confined to the parenchyma cells. The highly lignified thick-walled stone cells do not appear to alter (by microscopic examination, Sterling, 1954) during the maturation of the fruit, and since they represent over half the alcohol-insoluble material the changes (less the relatively constant amount from the stone cells) in the polysaccharides of the cell wall are even more striking than the above figures indicate. This is in agreement with microscopic examinations which show that when the fruit are overripe the walls of the parenchyma cells become extremely thin and tenuous, and in some areas may separate and collapse.

SUMMARY

1. The cell-wall material of the Conference pear that is insoluble in ethanol was divided into fractions by extraction with boiling water and cold alkali. Each fraction was hydrolysed and the mixture of sugars separated on a paper chromatogram and estimated colorimetrically.

2. It is assumed that each sugar in the hydrolysis mixtures corresponded to a single polysaccharide without regard to the fraction from which it was derived, and the results have been interpreted in terms of the constituent polysaccharides of the cell wall. The changes that occur in the amounts of these single-sugar polysaccharides (xylan, araban, polygalacturonic acid, galactan and cellulose) have been followed during the storage of the pear fruit at + 15° and + 5°.

3. Changes in the amount of the total cell wall and the fractions into which it is conveniently divided, 'pectin', 'hemicellulose', 'cellulose', are a reflexion of the separate changes in the amounts of the individual polysaccharides. The results indicate that the cell wall of the pear appears to be in dynamic equilibrium with the cytoplasm, and polysaccharides are both broken down and synthesized during the physiological changes which take place during ripening.

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REFERENCES

Barnell, H. S. (1943). *Ann. Bot., Lond.*, **7**, 297.
Beavan, G. H., Hirst, E. L. & Jones, J. K. N. (1939). *J. chem. Soc.* p. 1865.
Breddy, L. J. & Jones, J. K. N. (1945). *J. chem. Soc.* p. 738.
Chanda, S. K., Hirst, E. L. & Percival, E. G. V. (1951). *J. chem. Soc.* p. 1240.
Dreywood, R. (1946). *Industr. Engng Chem.* (*Anal.*), **18**, 499.
Echevin, R. (1941). *C.R. Acad. Sci., Paris*, **213**, 458.
Heuser, E. & Jayme, G. (1923). *J. prakt. Chem.* **105**, 232.
Hickinbottom, W. J. (1936). *Reactions of Organic Compounds*, p. 110. London: Longmans, Green and Co. Ltd.
Hirst, E. L. (1942). *J. chem. Soc.* p. 70.
Hirst, E. L., Isherwood, F. A., Jermyn, M. A. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 182.
Hirst, E. L. & Jones, J. K. N. (1938). *J. chem. Soc.* p. 1174.
Hirst, E. L. & Jones, J. K. N. (1939). *J. chem. Soc.* pp. 452, 454.
Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Jermyn, M. A. & Tomkins, R. G. (1950). *Biochem. J.* **47**, 437.
Kidd, F. & West, C. (1939). *New Phytol.* **38**, 105.
Kidd, F., West, C., Griffiths, D. G. & Potter, N. A. (1940). *Ann. Bot., Lond.*, (*N.S.*), **4**, 1.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Ritter, G. J., Seborg, R. M. & Mitchell, R. L. (1932). *Industr. Engng Chem.* (*Anal.*), **4**, 202.
Schulze, E. (1892). *Hoppe-Seyl. Z.* **16**, 387.
Smith, W. W. (1935). *Plant Physiol.* **10**, 587.
Sterling, C. (1954). *Food Res.* **19**, 433.
Wager, H. G. (1954). *Analyst*, **79**, 34.
Wise, L. E., Murphy, M. & D'Addieco, A. A. (1946). *Paper Tr. J.* **122**, 43.

The Synthesis of Indole by Washed Cell Suspensions of *Escherichia coli*

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The biosynthesis of the aromatic amino acids by micro-organisms has been mainly investigated by studies on the nutrition of normal and mutant cells and the accumulation of intermediate compounds in culture media. Recently several of the reactions concerned in aromatic synthesis in *Escherichia* and *Aerobacter* have been examined in detail with cell-free extracts (Kalan & Srinivasan, 1955; Mitsuhashi & Davis, 1954; Yaniv & Gilvarg, 1955; Yanofsky, 1955).

During an investigation of the formation of indole by bacteria it was found that washed-cell suspensions of certain mutants of *Aero. aerogenes* and *Esch. coli* would form aromatic compounds from glucose and ammonium salts. The formation of indole, anthranilic acid and an unidentified indole-like compound has been briefly reported by Gibson, Jones & Teltscher, 1955. This paper concerns the formation of indole.

MATERIALS AND METHODS

Strains of Esch. coli *used.* These (Table 1) were mutant strains derived originally from *Esch. coli* 518. They were obtained after ultraviolet irradiation and penicillin selection by the technique described by Davis (1948). Strains were maintained by serial subculture on heart-infusion agar.

Glassware and chemicals. Glassware was cleaned with a chromic acid cleaning mixture. The chemicals were the purest obtainable commercially; no attempt was made to purify them further.

Media. Two media were used for the routine preparation of cell suspensions.

(*a*) *Heart-infusion agar.* To 1 l. of tap water were added 500 g. of minced defatted ox heart, 10 g. of Evans peptone and 5 g. of NaCl. After steaming for 1 hr. the broth was filtered, the pH adjusted to 8·4, and the liquid autoclaved (15 lb./in.^{2} for 20 min.) and re-filtered. Agar (1·5%) was added, and after autoclaving at 15 lb./in.^{2} for 20 min. to dissolve the agar the pH was adjusted to 7·2–7·4. The medium was then autoclaved at 15 lb./in.^{2} for 20 min.

(*b*) *Synthetic medium.* The medium used was that described by Davis & Mingioli (1950). Glucose was added with the other ingredients before autoclaving. Double-strength liquid medium dispensed in 7·5 ml. volumes was sterilized at 10 lb./in.^{2} for 10 min. and added aseptically to an equal volume of sterile molten 3% agar.

Preparation and use of cell suspensions. Agar plates (4 in. diam.) were inoculated by spreading over them 0·2 ml. of a light (approx. 4×10^8 cells/ml.) suspension of organisms in distilled water. The inoculum was taken from a heart-

Table 1. *Strains of* Esch. coli

Mutant no.	Strain	Growth requirement*	Parent strain
—	518	None	—
1	7–4	Tryptophan	518
2	T2–15	Tryptophan (10^{-4} M, DL) plus serine (10^{-3} M, DL) or plus glycine (10^{-3} M)	7–4

* Supplement needed for growth in minimal medium (Davis & Mingioli, 1950), and concentration used during present experiments.